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DOXC-class 2-oxoglutarate-dependent dioxygenase in safflower: Gene characterization, transcript abundance, and correlation with flavonoids

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ABSTRACT

DOXC is one of the 2-oxoglutarate-dependent dioxygenases (2OGDs) classes, which is involved in the biosynthesis of colorful flavonoids and plant hormones. Safflower (*Carthamus tinctorius* L.) is a commonly used plant with flavonoids. Herein, we focused on analyzing the characterization, transcript abundance, and correlation of DOXC-class 2OGDs in safflower with flavonoids for the first time. TBLASTN analysis showed that they were implicated in the biosynthetic pathway of ethylene (DOXC1 and DOXC2), indole-3-acetic acid (DOXC3, DOXC4, and DOXC5), flavonoids (DOXC6, DOXC7, and DOXC8), and scopolamine (DOXC9 and DOXC10). The transcript abundance of DOXC1, DOXC2, DOXC6, DOXC7, and DOXC10 response to methyl jasmonate (MeJA) in two safflower lines (orange-yellow-flowered safflower line with major quinochalcones and minor flavonols, white-flowered safflower line with major flavonols and without quinochalcones) had converse transcript level accumulation. Coexpression analyses displayed that the transcript level of DOXC1, DOXC2 and DOXC8 was positively correlated with hydroxysafflor yellow A, a major quinochalcone in orange-yellow-flowered safflower line. DOXC3, DOXC4, DOXC5, DOXC7, and DOXC9 displayed a marked positive correlation with kaempferol-3-*O*-β-D-glucoside, a main flavonols in white-flowered safflower line. The response models of DOXC3, DOXC4, DOXC5 and DOXC9 showed amazing similarity in different safflower line, but the correlations with flavonoids were actually quite distinct. In orange-yellow-flowered safflower line, they might be implicated in the rutinoside modification of kaempferol and quercetin, but their functions were different and diversity in white-flowered safflower line. In sum, ten DOXC-class 2OGDs from safflower displayed different sequence features. Simultaneously, their expression pattern in two safflower lines in response to MeJA treatment, as well as their correlation with flavonoids, was different. These results implied the diversification of DOXC-class 2OGDs is partly responsible for the diversity and complexity of specialized metabolites in different phenotypes and chemotypes of safflower lines.

1. Introduction

In plants, the variety and complexity of phytochemicals resulted from the various functions of metabolic enzymes involved in the metabolic pathway. Among these diverse enzymes, oxygenases performing the oxidative/hydroxylated modification of specialized metabolites play apivotal roles, especially 2-oxoglutarate-dependent dioxygenases (2OGDs; Martens et al., 2003; Nelson and Werck Reichhart, 2011). 2OGDs are characterized by the 2-oxoglutarate-binding domain and the ferrous iron-binding domain, and their catalyzed reaction requires 2-oxoglutarate, molecular oxygen, and ferrous iron [Fe(II)] to catalyze the oxidation of a substrate (Farrow and Facchini, 2014). More than 100 putative 2OGD genes from *Arabidopsis* and rice have been identified and divided into each of the gene superfamilies (Yosuke et al., 2014). The active sites and reaction mechanisms of anthocyanidin synthase and the enzymes involved in ethylene biosynthetic pathway have been uncovered by crystal structure analysis (Holdsworth et al., 1987; Wilmouth et al., 2002). A comprehensive analysis of the 2OGD superfamily in six model plants, namely, *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Picea abies*, *Oryza sativa*, and *Arabidopsis thaliana*, has been reported, which pointed out that the evolution and variety of 2OGDs partly contributed to the forming of the vast structural diversity and complexity of idiosyncratic metabolites and that 2OGDs could be divided into three large classes (designated as DOXA, DOXB, and DOXC). Among the three classes, the DOXC class is involved in the biosynthesis

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of colorful flavonoids and plant hormones, DNA demethylation, as well as proline hydroxylation (Yosuke et al., 2014). Although the amino acid sequence of multiple 2OGDs is conserved in the carboxyl terminus, the 2OGDs in the DOXC class are extremely variable.

Safflower (*Carthamus tinctorius* L.) is a multipurpose crop used for its edible oil as well as its industrial and medicinal applications (Golkar et al., 2010). As a medically used plant, quinochalcones and flavonol are witnessed as the characterized and main constituents of safflower (Zhou et al., 2014; Tu et al., 2015). In recent years, much attention has been paid to the genetic diversity, molecular function, and genomic structure of safflower (Barati and Arzani, 2012; Golkar et al., 2011; Usha Kiran et al., 2015). With the use of sequencing technology, including expressed sequence tag (EST) analysis and next-generation sequencing (NGS), a rapid increase in the number of genes from safflower has been annotated and identified (Pearl et al., 2014; Ambreen et al., 2015). Therefore, the prediction of the function of multiple genes from safflower has become a matter of concern. Thus far, the genes encoding isochorismate synthase and cinnamate 4-hydroxylase in safflower have been cloned and characterized (Sadeghi et al., 2013; Dehghan et al., 2014). The characterization and functional expression of microsomal oleoylphosphatidyl cholinede saturase family (FAD2) have been revealed (Cao et al., 2013). Nonetheless, our understanding of genes from safflower still remains lacking. Considering the importance of safflower and the little knowledge of DOXC-class 2OGD genes in safflower that is available, we aimed to perform the inventory and sequence analysis of the 10 DOXC-class 2OGD genes from safflower. We also aimed to analyze the transcript abundance of 10 DOXC-class 2OGD genes in two safflower lines in response to methyl jasmonate (MeJA) treatment, along with their correlation with flavonoids. The results and analysis from this study will help gain insight into the potential function of the 2OGD superfamily in different safflower lines.

2. Materials and methods

2.1. Plant materials

Two safflower lines, namely, ZHH0119 line that possessed orange-yellow flower with major quinochalcones and minor flavonols, and XHH007 line that possessed white flower with flavonols but no quinochalcones, were planted in the greenhouse at a school of Second Military Medical University (Shanghai, China). The mean temperature was approximately 25 °C with regular irrigation and illumination (16 h). Fresh flowers were collected, frozen immediately in liquid nitrogen, and stored at −80 °C for RNA isolation.

2.2. Isolation and cloning of DOXC-class 2OGD genes

To obtain the full-length cDNA sequence of DOXC class in safflower, 5′- and 3′-rapid amplification of cDNA ends (RACE) experiments were conducted using SMART-RACE cDNA Amplification kit (Clontech, USA). Gene-specific primers used for cloning the DOXC class were designed based on DOXC-class fragment from the EST databases of safflower constructed by our laboratory (data not published; Supplementary file 1). The amplified product of 5′- and 3′-RACE was subcloned in pMD-19 vector (Takara, Dalian, China) for sequencing. DOXC-class-like cDNAs were amplified by polymerase chain reaction (PCR) according to the result of the sequence assembly of 5′- and 3′-RACE. The primer pairs were described in Supplementary file 1. The amplified products were cloned into pMD-19 vector and then sequenced for verification.

2.3. Sequence analysis and alignment

All the DOXC-class-like cDNA sequences obtained by PCR were analyzed with regard to their sequence characteristics. The open reading frames (ORFs) of these 2OGDs-like cDNA were uncovered on the National Center for Biotechnology Information (NCBI) Open Reading Frame Finder (ORF Finder, http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The ExPASyProtParam tool (http://web.expasy.org/protparam/) was applied to predict the theoretical isoelectric points, mass values, and molecular formula for these 2OGDs-like proteins. To identify the conserved motifs of the DOXC-class protein, their deduced amino acid sequences were aligned with DOXC-class proteins from other plants using DNAMAN software v8.0 software. PSORT server (http://www.genscript.com/psort/wolf_psort.html) was used to make a prediction in the presence of N-terminal signal peptides and protein subcellular localization. All the analyses were carried out with default settings.

2.4. Analysis of the secondary and tertiary structures of DOXC-class 2OGDs from safflower

NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/) was used in the analysis of the secondary structures of DOXC class using default parameters. The prediction of the tertiary structures of 2OGDs was performed on Phyre2 with intensive modeling. Both analyses were used in the amino acid sequences of 2OGDs as the target sequences.

2.5. Phylogenetic tree constructions

To study the evolutionary relationship among DOXC-class 2OGDs, the predicted amino acid sequences of DOXC-class 2OGDs of safflower and other plants were aligned using ClustalX1.83 and then phylogenetic trees were constructed by MEGA5.10 using the neighbor-joining (NJ) method and 1000 bootstrap replicates.

2.6. MeJA treatment

MeJA (100 μM, Sigma-Aldrich) solution was sprayed to the healthy flowers of the ZHH0119 and XHH007 lines that opened on the first day, which was defined as the day when the corolla protruded from the sepals and then enclosed with plastic bags. In the control group, the flowers were sprayed with same solution without MeJA and covered with plastic bags. After 0, 3, 6, and 12 h of handling, plastic bags were removed and at least three samples of flowers at four time points were collected and frozen immediately in liquid nitrogen and stored in −80 °C freezers.

2.7. Quantitative real-time PCR (qRT-PCR) analysis of DOXC-class 2OGDs

Flowers of the ZHH0119 and XHH007 lines were harvested after 0, 3, 6, and 12 h for transcript level analysis of 2OGDs. Total RNA of flowers was isolated using TransZol reagents (TransGen Biotech, Shanghai, China) following the manufacturer's instruction manual. RNA integrity was examined by running 1.0% (w/v) agarose gel and by detecting the A260/280 ratio. Quantification was performed on Biomatre 3S (Thermo Scientific). Primers used for qRT-PCR are also listed in Supplementary file 1. The first-strand cDNA was reverse transcribed following the TransScript First-Strand cDNA Synthesis SuperMix User Manual (TransGen Biotech, Beijing, China). qRT-PCR was performed with ABI7500 Real-Time PCR Detection System (Applied Biosystems). The PCR procedure was as follows: holding for 3 min at 95 °C followed by 35 cycles of denaturation for 10 s at 95 °C, annealing for 20 s at 58 °C, and elongation for 30 s at 72 °C. Primer specificity (single product of expected length) was verified by analysis by running 1.0% agarose gel and by melting curve analysis with procedure of 60 °C for 1 min and 95 °C for 30 s. All PCR reactions consisted of three technical replicates. The transcript level of each 2OGD was normalized to Ct60s (KJ634810) as the reference gene and compared to their transcript level at 0 h using the $2^{-\Delta\Delta Ct}$ method.